



Endothelial barrier protection by local anesthetics: ropivacaine and lidocaine block tumor necrosis factor- α -induced endothelial cell Src activation

Piegeler, Tobias ; Votta-Velis, E Gina ; Bakhshi, Farnaz R ; Mao, Mao ; Carnegie, Graeme ; Bonini, Marcelo G ; Schwartz, David E ; Borgeat, Alain ; Beck-Schimmer, Beatrice ; Minshall, Richard D

Abstract: BACKGROUND: Pulmonary endothelial barrier dysfunction mediated in part by Src-kinase activation plays a crucial role in acute inflammatory disease. Proinflammatory cytokines, such as tumor necrosis factor- α (TNF), activate Src via phosphatidylinositol 3-kinase/Akt-dependent nitric oxide generation, a process initiated by recruitment of phosphatidylinositol 3-kinase regulatory subunit p85 to TNF-receptor-1. Because amide-linked local anesthetics have well-established anti-inflammatory effects, the authors hypothesized that ropivacaine and lidocaine attenuate inflammatory Src signaling by disrupting the phosphatidylinositol 3-kinase-Akt-nitric oxide pathway, thus blocking Src-dependent neutrophil adhesion and endothelial hyperpermeability. METHODS: Human lung microvascular endothelial cells, incubated with TNF in the absence or presence of clinically relevant concentrations of ropivacaine and lidocaine, were analyzed by Western blot, probing for phosphorylated/activated Src, endothelial nitric oxide synthase, Akt, intercellular adhesion molecule-1, and caveolin-1. The effect of ropivacaine on TNF-induced nitric oxide generation, co-immunoprecipitation of TNF-receptor-1 with p85, neutrophil adhesion, and endothelial barrier disruption were assessed. RESULTS: Ropivacaine and lidocaine attenuated TNF-induced Src activation (half-maximal inhibitory concentration [IC₅₀] = 8.611×10^{-6} M for ropivacaine; IC₅₀ = 5.864×10^{-6} M for lidocaine) and endothelial nitric oxide synthase phosphorylation (IC₅₀ = 7.572×10^{-6} M for ropivacaine; IC₅₀ = 6.377×10^{-6} M for lidocaine). Akt activation (n = 7; P = 0.006) and stimulus-dependent binding of TNF-receptor-1 and p85 (n = 6; P = 0.043) were blocked by 1 nM of ropivacaine. TNF-induced neutrophil adhesion and disruption of endothelial monolayers via Src-dependent intercellular adhesion molecule-1- and caveolin-1-phosphorylation, respectively, were also attenuated. CONCLUSIONS: Ropivacaine and lidocaine effectively blocked inflammatory TNF signaling in endothelial cells by attenuating p85 recruitment to TNF-receptor-1. The resultant decrease in Akt, endothelial nitric oxide synthase, and Src phosphorylation reduced neutrophil adhesion and endothelial hyperpermeability. This novel anti-inflammatory "side-effect" of ropivacaine and lidocaine may provide therapeutic benefit in acute inflammatory disease.

DOI: <https://doi.org/10.1097/ALN.0000000000000174>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-107147>

Journal Article

Published Version

Originally published at:

Piegeler, Tobias; Votta-Velis, E Gina; Bakhshi, Farnaz R; Mao, Mao; Carnegie, Graeme; Bonini, Marcelo G; Schwartz, David E; Borgeat, Alain; Beck-Schimmer, Beatrice; Minshall, Richard D (2014). Endothelial

barrier protection by local anesthetics: ropivacaine and lidocaine block tumor necrosis factor- α -induced endothelial cell Src activation. *Anesthesiology*, 120(6):1414-1428.
DOI: <https://doi.org/10.1097/ALN.000000000000174>

Endothelial Barrier Protection by Local Anesthetics

Ropivacaine and Lidocaine Block Tumor Necrosis Factor- α -induced Endothelial Cell Src Activation

Tobias Piegeler, M.D., E. Gina Votta-Velis, M.D., Ph.D., Farnaz R. Bakhshi, Ph.D., Mao Mao, M.S., Graeme Carnegie, Ph.D., Marcelo G. Bonini, Ph.D., David E. Schwartz, M.D., Alain Borgeat, M.D., Beatrice Beck-Schimmer, M.D., Richard D. Minshall, Ph.D.

ABSTRACT

Background: Pulmonary endothelial barrier dysfunction mediated in part by Src-kinase activation plays a crucial role in acute inflammatory disease. Proinflammatory cytokines, such as tumor necrosis factor- α (TNF α), activate Src *via* phosphatidylinositol 3-kinase/Akt-dependent nitric oxide generation, a process initiated by recruitment of phosphatidylinositol 3-kinase regulatory subunit p85 to TNF-receptor-1. Because amide-linked local anesthetics have well-established anti-inflammatory effects, the authors hypothesized that ropivacaine and lidocaine attenuate inflammatory Src signaling by disrupting the phosphatidylinositol 3-kinase–Akt–nitric oxide pathway, thus blocking Src-dependent neutrophil adhesion and endothelial hyperpermeability.

Methods: Human lung microvascular endothelial cells, incubated with TNF α in the absence or presence of clinically relevant concentrations of ropivacaine and lidocaine, were analyzed by Western blot, probing for phosphorylated/activated Src, endothelial nitric oxide synthase, Akt, intercellular adhesion molecule-1, and caveolin-1. The effect of ropivacaine on TNF α -induced nitric oxide generation, co-immunoprecipitation of TNF-receptor-1 with p85, neutrophil adhesion, and endothelial barrier disruption were assessed.

Results: Ropivacaine and lidocaine attenuated TNF α -induced Src activation (half-maximal inhibitory concentration [IC₅₀] = 8.611×10^{-10} M for ropivacaine; IC₅₀ = 5.864×10^{-10} M for lidocaine) and endothelial nitric oxide synthase phosphorylation (IC₅₀ = 7.572×10^{-10} M for ropivacaine; IC₅₀ = 6.377×10^{-10} M for lidocaine). Akt activation ($n = 7$; $P = 0.006$) and stimulus-dependent binding of TNF-receptor-1 and p85 ($n = 6$; $P = 0.043$) were blocked by 1 nM of ropivacaine. TNF α -induced neutrophil adhesion and disruption of endothelial monolayers *via* Src-dependent intercellular adhesion molecule-1- and caveolin-1-phosphorylation, respectively, were also attenuated.

Conclusions: Ropivacaine and lidocaine effectively blocked inflammatory TNF α signaling in endothelial cells by attenuating p85 recruitment to TNF-receptor-1. The resultant decrease in Akt, endothelial nitric oxide synthase, and Src phosphorylation reduced neutrophil adhesion and endothelial hyperpermeability. This novel anti-inflammatory “side-effect” of ropivacaine and lidocaine may provide therapeutic benefit in acute inflammatory disease. (**ANESTHESIOLOGY 2014; 120:1414-28**)

VASCULAR inflammation is thought to underlie the high morbidity and mortality of diseases such as acute lung injury, acute respiratory distress syndrome (ARDS), diabetes mellitus, and cancer.¹⁻⁵ Therapeutic options are limited to the treatment of symptoms (*e.g.*, lung-protective ventilation in patients with ARDS) rather than the cause of endothelial barrier dysfunction.⁶ Vascular hyperpermeability and endothelial barrier dysfunction play key roles in the pathogenesis of vascular injury due to inflammation.^{6,7} The proinflammatory cytokine tumor necrosis factor- α (TNF α) activates c-Src protein tyrosine kinase (Src) and increases endothelial permeability.⁸ Although the exact mechanism of TNF α -induced Src activation is not completely understood, it has been

What We Already Know about This Topic

- The proinflammatory cytokine tumor necrosis factor- α activates Src tyrosine kinase and increases endothelial permeability. Whether some anti-inflammatory effects of amide local anesthetics (such as those observed in models of acute lung injury) are due to inhibition of signaling upstream or downstream of Src remains unknown.

What This Study Tells Us That Is New

- Using human lung microvascular endothelial cells, it was shown that ropivacaine and lidocaine attenuated tumor necrosis factor- α -induced neutrophil adhesion and endothelial hyperpermeability *via* a reduction of Akt, endothelial nitric oxide synthase, and Src activation.

Submitted for publication June 10, 2013. Accepted for publication January 8, 2014. From the Department of Anesthesiology (T.P., E.G.V.-V., D.E.S., R.D.M.), Department of Pharmacology (F.R.B., M.M., G.C., M.G.B., R.D.M.), Department of Medicine (M.G.B.), and Center for Lung and Vascular Biology (R.D.M.), University of Illinois Hospital and Health Sciences System, Chicago, Illinois; Institute of Anesthesiology (T.P.), University Hospital Zurich, Zurich, Switzerland; Institute of Physiology, Zurich Center for Integrative Human Physiology (B.B.-S.), University of Zurich; Department of Anesthesiology (A.B.), Balgrist University Hospital Zurich, Zurich, Switzerland; and Department of Anesthesiology (E.G.V.-V.), Jesse Brown VA Medical Center, Chicago, Illinois.

Copyright © 2014, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2014; 120:1414-28

demonstrated that endothelial nitric oxide synthase (eNOS)-mediated nitric oxide production downstream of phosphatidylinositol 3-kinase (PI3K)-dependent activation of Akt^{9–11} and CaM kinase II induce Src activation.^{10,12} TNF α -induced PI3K activation is thought to be initiated by recruitment of PI3K regulatory subunit p85 to TNF-receptor-1 (TNF-R1) upon agonist binding.¹³ Downstream signaling of activated Src includes phosphorylation of intercellular adhesion molecule-1 (ICAM-1) and caveolin-1, which have both been shown to contribute to the development of the endothelial injury and inflammatory hyperpermeability.^{14–17}

Experimental evidence indicates that amide-linked local anesthetics such as ropivacaine and lidocaine exhibit beneficial properties during the pathogenesis of acute lung injury: they were proven to attenuate experimental (endotoxin-induced) lung inflammation *in vivo* and *in vitro*^{18,19} as well as neutrophil-dependent acute lung injury induced by *N*-formyl-L-leucine-methionyl-L-phenylalanine^{20,21}. In addition, we recently demonstrated inhibition of TNF α -induced Src activation by ropivacaine and lidocaine in a human lung cancer cell line *in vitro*.²² We therefore hypothesized that amide-linked local anesthetics, such as ropivacaine and lidocaine, may attenuate inflammatory Src signaling and subsequent ICAM-1 and caveolin-1 phosphorylation by disrupting the PI3K–Akt–nitric oxide signaling pathway, thus blocking Src-dependent vascular inflammation and hyperpermeability of the endothelium. The results of the current study indicate a novel anti-inflammatory mechanism by which ropivacaine and lidocaine may minimize endothelial injury during the pathogenesis of acute lung injury or ARDS.

Materials and Methods

Cell Culture

Human lung microvascular endothelial cells (HLMVECs; Lonza, Walkersville, MD) were cultured on dishes coated with 0.2% gelatin (Sigma-Aldrich, St. Louis, MO) in VascuLife[®] Basal Medium (Lifeline Cell Technology, Walkersville, MD) supplemented with the VascuLife[®] VEGF-Mv LifeFactors Kit containing 5% fetal bovine serum, 10 mM of L-glutamine, 5 ng/ml of recombinant vascular endothelial growth factor, 5 ng/ml of recombinant epithelial growth factor, 5 ng/ml of fibroblast growth factor, 15 ng/ml of insulin-like growth factor-1, 1 μ g/ml of hydrocortisone hemisuccinate, 0.75 U/ml of heparin, 50 μ g/ml of ascorbic acid (Lifeline Cell Technology), and 1% penicillin–streptomycin (Gibco Invitrogen, Carlsbad, CA). Experiments were performed with 70 to 100% confluent cells from passages 5 to 11. Before the experiments, cells were grown in a serum-free media (VascuLife[®] Basal Medium) for 16 h. All cells were maintained in 5% carbon dioxide–95% room air in a water-jacketed 37°C incubator.

Experimental Procedures

Cell monolayers were incubated with TNF α (Gibco Invitrogen) at a concentration of 20 ng/ml diluted in VascuLife[®]

Basal Medium for 20 min for Western blot analysis or for 10 min for immunoprecipitation. Ropivacaine 0.5% (Naropin[®]; APP Pharmaceuticals, Schaumburg, IL) or lidocaine 2% (APP Pharmaceuticals) were diluted with VascuLife[®] Basal Medium (1 pM to 100 μ M) and were incubated for the same time period as TNF α . If tested together on the same cell monolayer, ropivacaine and TNF α were added to the medium simultaneously.

Cell Harvest and Lysis

After the indicated incubation times, HLMVECs were washed once with ice-cold phosphate-buffered saline (Gibco Invitrogen), scraped from the bottom of the plate, collected, and lysed with radio-immunoprecipitation buffer (Boston Bioproducts, Ashland, MA) supplemented with protease inhibitor cocktail, 200 mM of phenylmethylsulfonyl fluoride, 1 mM of EDTA, 1 mM of sodium fluoride, and 1 mM of sodium orthovanadate (all from Sigma-Aldrich) as previously described.^{22,23}

Determination of Total Protein Concentration

Whole cell lysates prepared as described above were subject to total protein concentration measurement using the DC Protein Assay Kit (BioRad, Hercules, CA) in accordance with the manufacturer's instructions. Immunoglobulin G was used for the generation of a standard curve.

Immunoprecipitation

To assess the association of TNF-R1 and p85 subunit of PI3K, HLMVECs were washed once with ice-cold phosphate-buffered saline (Gibco Invitrogen) and lysed using a buffer consisting of 20 mM of Tris-HCl (pH 7.4), 150 mM of NaCl, 1.2% Triton X-100, protease inhibitor cocktail, 200 mM of phenylmethylsulfonyl fluoride, 1 mM of EDTA, 1 mM of sodium fluoride, and 1 mM of sodium orthovanadate for 20 min during gentle rocking of the dish at 4°C as previously described.¹³ Lysates were then incubated with magnetic sheep anti-mouse-IgG-coated Dynabeads (Gibco Invitrogen) for 1 h at 4°C that had previously been incubated for 30 min at 4°C with nonspecific mouse immunoglobulin G (the negative control) or anti-TNF-R1 monoclonal antibody (both from Santa Cruz Biotechnology). The lysate-bead suspensions were then placed in a magnetic particle concentrator (Invitrogen, Carlsbad, CA) and the pellet washed thrice with ice-cold (4°C) Ca²⁺- and Mg²⁺-free phosphate-buffered saline (Gibco Invitrogen) that also included the same supplements as the lysis buffer (see Materials and Methods, Cell harvest and lysis). Laemmli sample buffer ($\times 2$; BioRad) was added together with dithiothreitol (final concentration of 30 mM; Sigma-Aldrich). The suspension was then boiled for 5 min to elute the protein complexes from the beads. After removal of the beads by magnetic separation, samples were directly loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and Western blot analysis or stored at –80°C until further use.

Western Blot Analysis

Western blotting of whole cell lysates was carried out as previously described.^{22,23} Antibodies against pY419 Src, total Src, and p85 were purchased from Cell Signaling Technologies (Danvers, MA), for pY512 ICAM-1, total ICAM-1, glyceraldehyde 3-phosphate dehydrogenase, and TNF-R1 (both rabbit and mouse origin) from Santa Cruz Biotechnology, and for pY14 caveolin-1, total caveolin-1, and β -actin from BD Biosciences (Franklin Lakes, NJ).

In Vitro Src Kinase Assay

Src kinase activity in the absence or presence of different concentrations of ropivacaine (1 nM to 1 mM) or the Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine (PP2, 10 μ M; from Calbiochem Millipore, Billerica, MA) was assessed using an assay previously described by Hastie *et al.*,²⁴ which uses the incorporation of radioactively labeled [γ -³²P]-adenosine triphosphate (Perkin Elmer, Waltham, MA) into a specific substrate peptide. Both purified, active Src and its specific substrate peptide were purchased from Millipore. Radioactivity, expressed as counts per minute (cpm), was measured in a γ -scintillation counter (Beckman Coulter, Indianapolis, IN).

PI3K In Vitro Kinase Assay

Assessment of PI3K activity *in vitro* was carried out with a commercially available PI3K activity/inhibitor assay kit (Millipore) according to the manufacturer's instructions. The assay is based on the principle of an enzyme-linked immunosorbent assay and detects biotinylated phosphatidylinositol (3,4,5)-triphosphate (B-PIP3) captured with general receptor for phosphoinositides-1 bound to plates coated with glutathione using a streptavidin-horse radish peroxidase conjugate and colorimetric readout at 450 nm. PI3K converts biotinylated phosphatidylinositol (4,5)-bisphosphate (PIP2) to PIP3. The generated PIP3 competes with added B-PIP3 for glutathione binding. A high signal in the colorimetric readout therefore indicates low levels of generated PIP3, thus low enzyme activity. We evaluated different concentrations of ropivacaine (Sigma-Aldrich; solved in dimethyl sulfoxide, final concentration of 1 nM, 1 μ M, or 100 μ M), as potential PI3K inhibitors and used wortmannin (100 nM), a known PI3K inhibitor, as a positive control. Four different isoforms of the p110 catalytic subunit of PI3K (α , β , γ , δ) were tested separately.

Measurement of Nitrite Ion Accumulation and Nitric Oxide Production in HLMVEC

Chemiluminescence measurement of nitrite ion accumulation in cell culture supernatant, which is directly proportional to nitric oxide production, was conducted with a Sievers[®] NOA 280i high-performance liquid chromatography with fluorescence detection (General Electric, Boulder, CO) as previously described.¹¹ HLMVEC monolayers were treated with TNF α (20 ng/ml) in the absence or presence of different concentrations of ropivacaine (1 nM, 1 μ M, or 100 μ M). For some experiments, the cells were

pretreated with the NOS inhibitor N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO; Cayman Chemical, Ann Arbor, MI) for 1 h before the application of TNF α .

Transendothelial Resistance

Human lung microvascular endothelial cells were grown to confluence on goldfilm electrodes (ECIS cultureware; Applied Biophysics, Troy, NY) coated with 0.2% gelatin. Electrical impedance across the monolayer was measured at 1 V, 4,000 Hz with the Electric Cell-Substrate Impedance Sensing system (Applied Biophysics) over time as previously described.²⁵ After an equilibration time of 30 min, TNF α was added with or without ropivacaine at a final concentration of 1 μ M. For some experiments, cells were treated with the NOS inhibitor L-NIO (Cayman Chemical) before the addition of TNF α . Impedance values were used to calculate resistance using ECIS software.

Isolation of Human Polymorphonuclear Leukocytes

Whole blood samples were drawn from cubital veins from healthy donors and anticoagulated with 1 ml acid-citrate-dextrose (Sigma-Aldrich) per 5 ml whole blood according to approved Institutional Review Board (University of Illinois at Chicago, Chicago, IL) protocol. Samples were suspended in a three-fold volume of ice-cold lysis medium containing 155 mM of ammonium chloride and 10 mM of potassium bicarbonate (both from Sigma-Aldrich) and kept on ice for 10 min. After brief centrifugation at 1,500 rpm and 4°C (Eppendorf Centrifuge 5804 R; Eppendorf, Hamburg, Germany), the supernatant was discarded and the cell pellet was resuspended in 2 ml of ice-cold lysis medium supplemented with 0.5% bovine serum albumin. The suspension was then kept on ice 15 min followed by another centrifugation at 1,500 rpm and 4°C. Again, the supernatant was discarded. The pellet was resuspended in 2 ml of ice-cold Dulbecco phosphate-buffered saline (Gibco Invitrogen) and layered on 2 ml of Ficoll Histopaque 1077 (Sigma-Aldrich). After 30 min of centrifugation at 1,500 rpm and 4°C with low brake, the supernatant was discarded and the pellet consisting of neutrophils was resuspended in Hank balanced salt solution (Gibco Invitrogen). Viability was 95% or more as assessed with Trypan blue staining (Sigma-Aldrich).

Neutrophil Adhesion Assay

Neutrophil adhesion to a monolayer of HLMVECs was assessed by using the CytoSelect[®] Leukocyte-Endothelium Adhesion Assay (Cell Biolabs, San Diego, CA) in accordance with the manufacturer's instructions.¹⁷ In brief, HLMVECs were grown in a 96-well plate (BD Biosciences) coated with 0.2% gelatin (Sigma-Aldrich). To increase ICAM-1 expression, all cells were subject to stimulation with *Escherichia coli* serotype 055:B5 lipopolysaccharide (Sigma-Aldrich) at a concentration of 4 μ g/ml for 4 h. Cells were then stimulated with TNF α in the absence or presence of ropivacaine at different concentrations (1 nM, 1 μ M, or 100 μ M) for 20 min before

addition of fluorescently labeled, freshly isolated human neutrophils (100,000 per well). After an incubation period of 30 min, nonadherent neutrophils were washed away and remaining cells were lysed; fluorescence of the lysate was measured at 485/535 nm (excitation/emission).

Determination of Half-maximal Inhibitory Concentration (IC_{50}) and Maximum Effect Size

The half-maximal inhibitory concentration (IC_{50}) is defined as the concentration of a drug where it exerts 50% of its maximum effect.²⁶ To determine these values for the effect of ropivacaine and lidocaine on TNF α -induced Src and eNOS activation, densitometry data obtained from Western blot experiments (mean \pm SD, normalized to values of TNF α alone, set as 1.0) were plotted against the (logarithmic) concentration of the local anesthetic used in the experiment. A concentration–response curve with a Hill slope of -1.0 was fitted by nonlinear regression analysis using the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{Log } IC_{50})})$$

Results are reported as values with 95% CI (for IC_{50} , top, and bottom). Nonlinear regression curve fitting was computed with GraphPad Prism software for Mac, Version 6 (GraphPad Software, La Jolla, CA).

Statistical Analysis

For Western blot, Src and PI3K activity assay, and transendothelial resistance (TER) data, normal distribution was assessed using a Shapiro–Wilk test. Normally distributed data were analyzed by one-way ANOVA with Bonferroni *post hoc* testing to correct for multiple testing and to keep the family-wise error rate less than 0.05. Not-normally distributed data were analyzed with nonparametric testing methods (Kruskal–Wallis tests) with Dunn *post hoc* correction.

Nitric oxide production and fluorescence from adhesion experiments were analyzed by using a two-way ANOVA with the local anesthetic and the absence or presence of TNF α as factors to be tested. Bonferroni *post hoc* testing was then used to detect significant differences between the different concentrations of ropivacaine in the absence or presence of TNF α (one family for all comparisons). Experimental data are presented as mean \pm SD. All tests were performed two-sided and nonblinded using GraphPad Prism software for Mac, Version 6 (GraphPad Software). A *P* value of less than 0.05 was considered to be statistically significant. A few single values (approximately 5 to 7) were lost to observation due to unfortunate and unexpected technical difficulties during the experiment.

Results

Ropivacaine and Lidocaine Inhibit the Activation of Src Tyrosine Kinase (Src) in HLMVEC but Do Not Act as Direct, Adenosine Triphosphate-competitive Src Inhibitors

We first assessed the effect of ropivacaine and lidocaine at a concentration of 1 nM on TNF α (20 ng/ml for 20 min)-induced

activation of Src in HLMVEC. Western blot analysis of active-site Src phosphorylation (phospho-tyrosine 419; pY419 Src) and total Src protein level in whole cell lysates (fig. 1A) indicated that TNF α treatment of cells increased Src activity 2.3-fold compared with untreated cells (2.3 ± 0.93 [mean \pm SD], $P = 0.006$, fig. 1Bi, and 2.3 ± 0.51 , $P = 0.004$, fig. 1Bii). The TNF α -induced response was then reduced by $50 \pm 9\%$ in presence of 1 nM of ropivacaine ($P = 0.035$; fig. 1Bi) and by $49 \pm 16\%$ by lidocaine (1 nM; $P = 0.034$), which in both cases brought the level of Src activation nearly to baseline values. Neither ropivacaine nor lidocaine, even at concentrations of 100 μ M, altered Src phosphorylation significantly compared with untreated cells (Kruskal–Wallis test; $P = 0.534$ for ropivacaine; $P = 0.946$ for lidocaine, data not shown).

To determine the IC_{50} of the observed inhibition of TNF α -induced Src activation by ropivacaine and lidocaine, another series of Western blot analyses treating HLMVEC with TNF α in the absence or presence of a broad range of concentrations of both ropivacaine and lidocaine (1 pM to 1 μ M) was conducted. Figure 1C shows the ratio of pY419 Src over total Src (mean \pm SD) plotted against the logarithmic concentration of either ropivacaine (fig. 1Ci) or lidocaine (fig. 1Cii) relative to TNF α alone. We then analyzed the data using nonlinear regression curve fitting. The results are summarized in table 1. The IC_{50} for TNF α -induced Src activation was 861 pM (95% CI, 79 pM to 9.4 nM) for ropivacaine and 586 pM (59.6 pM to 6.8 nM) for lidocaine. The maximum observed effect was 56% (95% CI, 68.9 to 43) for ropivacaine and 39% (55.8 to 23.3) for lidocaine.

To clarify whether ropivacaine blocks TNF α -induced Src activation as opposed to functioning as a competitive active-site Src inhibitor, we performed an *in vitro* Src kinase assay using radiolabeled [γ -³²P] adenosine triphosphate (fig. 1D). Purified active Src kinase (15 ng) was incubated with a Src-specific substrate peptide (250 μ M) in the absence or presence of either the Src inhibitor PP2 (10 μ M) or different concentrations of ropivacaine (1 nM to 1 mM). Only the Src inhibitor PP2 reduced Src activity in this setting ($P = 0.0008$); ropivacaine did not alter Src activity at any of the concentrations tested ($P = 1$ for all groups compared with positive control substrate + enzyme, fig. 1D), and thus is not a direct inhibitor of c-Src.

Ropivacaine (and Lidocaine) Block TNF α -induced eNOS Phosphorylation and Nitric Oxide Production in HLMVECs

The generation of nitric oxide can promote Src activation in response to different stimuli.¹⁰ We therefore assessed eNOS activation by Western blot analyses of active-site serine 1177 phosphorylation in HLMVEC lysates treated with TNF α in the absence or presence of 1 nM of ropivacaine or lidocaine. As shown in figure 2, A and B, TNF α treatment induced a 2.76 ± 1.39 -fold ($P = 0.002$) increase in eNOS phosphorylation (pS1177 eNOS) and this response to TNF α was reduced by $45 \pm 15\%$ in presence of 1 nM of ropivacaine ($P = 0.022$; fig. 2, Ai and Bi). Lidocaine at 1 nM concentration significantly reduced TNF α -induced eNOS phosphorylation (2.68 ± 0.66 ;

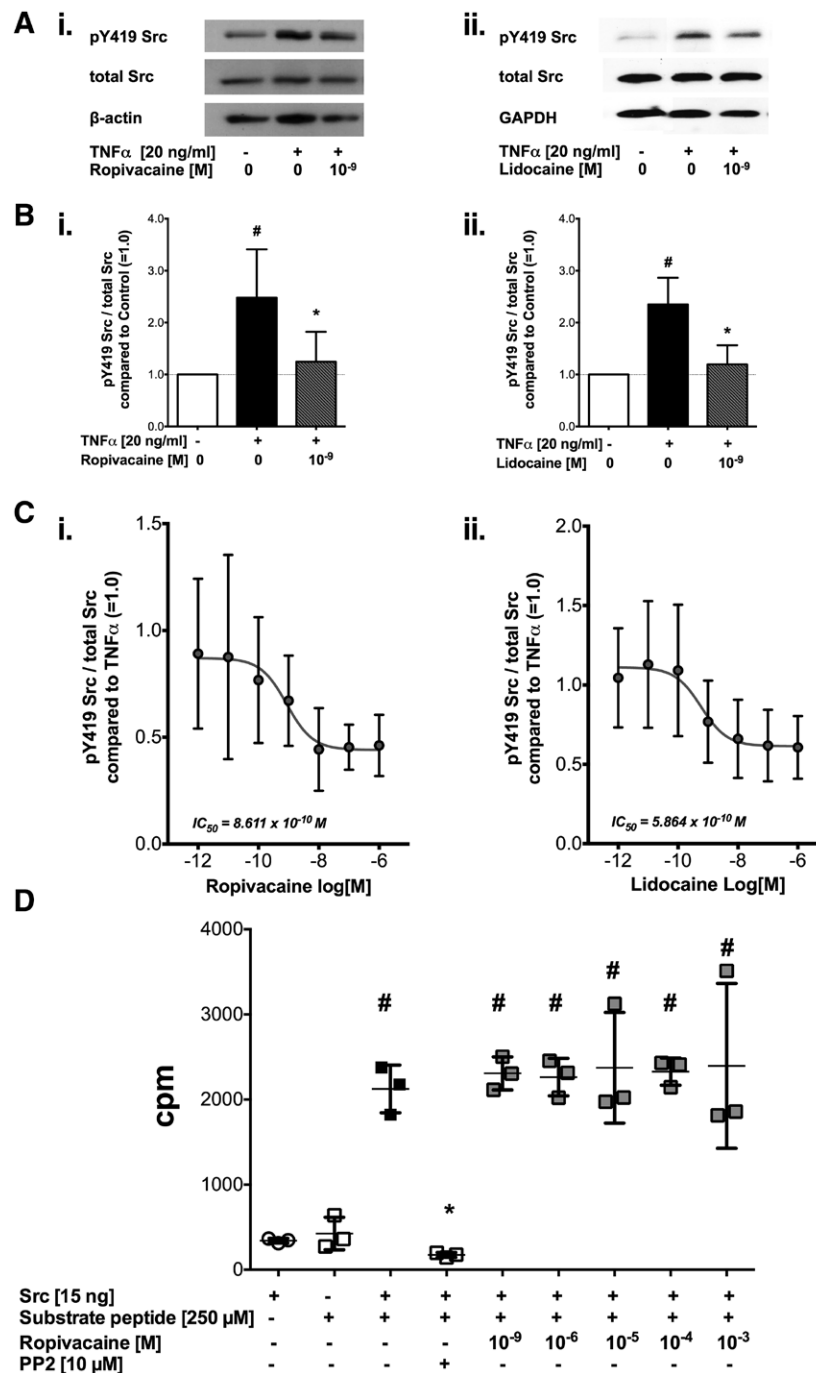


Fig. 1. Ropivacaine and lidocaine inhibit the activation of Src tyrosine kinase (Src) in human lung microvascular endothelial cells (HLMVECs). (A) Representative Western blots of Src phosphorylated at tyrosine 419 (pY419 Src, row 1), total Src (row 2), and β -actin (row 3) after treatment with tumor necrosis factor- α (TNF α) in the absence or presence of (i) ropivacaine (1 nM) or (ii) lidocaine for 20 min. (B) Quantitative analysis of densitometry of Western blots showing the ratio of pY419 Src over total Src in HLMVEC treated with TNF α (20 ng/ml) in the absence (black bar) or presence (striped gray bar) of (i) ropivacaine or (ii) lidocaine compared with untreated cells (white bar, set as 1.0). Data shown are mean \pm SD (n = 6 per group); #P < 0.05 versus untreated cells, *P < 0.05 compared with TNF α alone. (C) Dose-response curve of Western blot densitometry data showing the ratio of pY419 Src over total Src in HLMVEC treated with TNF α (20 ng/ml) in the absence or presence of (i) ropivacaine (1 pM to 1 μ M) or (ii) lidocaine (1 pM to 1 μ M) compared with TNF α alone (set as 1.0). Data shown are mean \pm SD (n = 5–6). (D) *In vitro* Src kinase activity. Purified, active Src kinase (15 ng) was incubated with radiolabeled [γ - ^{32}P] adenosine triphosphate, a specific substrate peptide (250 μ M) in the absence (black bar) or presence of either the Src inhibitor PP2 (10 μ M, striped gray bar) or different concentrations of ropivacaine (1 nM to 1 mM, gray bars). Data shown are mean \pm SD of radioactive counts per minute (cpm) as a measure of [γ - ^{32}P] adenosine triphosphate turnover and therefore Src enzyme activity (n = 3 per group); #P < 0.05 versus enzyme or substrate alone, *P < 0.05 compared with enzyme plus substrate. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

Table 1. Inhibitory Properties of Ropivacaine and Lidocaine on TNF α -induced Src and eNOS Phosphorylation

Drug	Enzyme	IC ₅₀ (M) (95% CI)	Bottom (95% CI)	Top (95% CI)
Ropivacaine	pY419 Src	8.611×10^{-10} (7.901×10^{-11} to 9.384×10^{-9})	0.441 (0.311–0.570)	0.870 (0.719–1.022)
	pS1177 eNOS	7.572×10^{-10} (1.177×10^{-10} to 4.872×10^{-9})	0.606 (0.411–0.802)	1.268 (1.08–1.456)
Lidocaine	pY419 Src	5.864×10^{-10} (5.964×10^{-11} to 5.765×10^{-9})	0.614 (0.462–0.767)	1.111 (0.942–1.279)
	pS1177 eNOS	6.377×10^{-10} (9.200×10^{-11} to 4.420×10^{-9})	0.456 (0.266–0.646)	1.198 (0.975–1.420)

IC₅₀ = half-maximal inhibitory concentration; pS1177 eNOS = endothelial nitric oxide synthase, phosphorylated at serine 1177 (active eNOS); pY419 Src = Src, phosphorylated at tyrosine 419 (active Src).

$P = 0.011$; fig. 2, Aii and Bi) by $49 \pm 32\%$ ($P = 0.022$; fig. 2, Aii and Bii). The dose–response relationship for inhibition of TNF α -induced eNOS phosphorylation by both ropivacaine and lidocaine (fig. 2C) revealed an IC₅₀ of 757 pM (95% CI, 117.7 pM to 4.9 nM; table 1) for ropivacaine, which had a maximal effect of 39.4% (58.9 to 19.8), whereas lidocaine showed an IC₅₀ of 638 pM (92 pM to 44.2 nM) and a maximum effect of 54% (74.4 to 35.4; table 1) in these experiments. Neither ropivacaine nor lidocaine, even at concentrations of 100 μ M, directly altered eNOS phosphorylation compared with untreated cells (Kruskal–Wallis test, $P = 0.972$ for ropivacaine; $P = 0.359$ for lidocaine, data not shown).

The effect of ropivacaine at different concentrations (1 nM, 1 μ M, and 100 μ M) on TNF α -induced (20 ng/ml) nitric oxide production in HLMVEC as compared with cells treated with the NOS-inhibitor L-NIO (20 μ M) was examined. Data analysis using two-way ANOVA with the presence of TNF α and the different experimental treatments (L-NIO or ropivacaine) as the factors to be tested revealed a significant impact of the presence of TNF α ($F(1, 31) = 40.35$; $P < 0.0001$) and the different drugs and doses on nitric oxide production by the cells ($F(4, 31) = 25.93$; $P < 0.0001$). A significant interaction term between the two factors ($F(4, 31) = 16.55$; $P < 0.0001$) was also found. *Post hoc* testing revealed significant differences between L-NIO and the different concentrations of ropivacaine when incubated together with TNF α compared with TNF α alone ($P < 0.0001$ for all comparisons). As shown in figure 2D, the TNF α -induced increase was completely abolished by pretreating the cells with L-NIO and 1 nM of ropivacaine. However, the presence of neither ropivacaine nor L-NIO had an effect on basal nitric oxide production ($P = 1$ for all comparisons; fig. 2D).

Ropivacaine Blocks TNF α -induced Activation of Akt Upstream of eNOS but Does Not Directly Inhibit PI3K Activity Upstream of Akt

To establish the mechanism by which ropivacaine blocks TNF α -induced eNOS and Src activation, we determined whether ropivacaine blocks TNF α -induced activation of Akt, an upstream kinase known to activate eNOS¹¹ via phosphorylation of serine 1177. HLMVECs exposed to ropivacaine (1 nM, 1 μ M) in the absence or presence of TNF α (20 ng/ml) for 20 min were subject to Western blot analysis, probing for “activated” Akt, phosphorylated at threonine 308 (pT308 Akt, the PI3K-dependent phosphorylation

site),²⁷ as well as total Akt which served as a loading control (fig. 3A). Densitometry analysis revealed that ropivacaine alone had no effect on basal Akt phosphorylation ($P = 0.878$, data not shown), whereas the TNF α -induced increase in Akt phosphorylation ($214 \pm 44\%$ of untreated cells; $P = 0.013$) was completely abolished by 1 nM of ropivacaine ($P = 0.005$; fig. 3B). As Akt activation is known to be induced by PI3K,^{11,28} we determined whether ropivacaine might act as a direct inhibitor of PI3K catalytic activity. PI3K activity *in vitro* was determined by enzyme-linked immunosorbent assay measurements of PIP3 generated by PI3K. In this particular assay, a *high* signal indicates poor competition with B-PIP3 and *low* enzyme activity. Different isoforms of p110 (α , β , γ , and δ), the catalytic subunit of PI3K, were incubated with PIP2 substrate in the absence or presence of different concentrations of either ropivacaine or wortmannin (100 nM), a known PI3K inhibitor.¹⁷ As shown in figure 3C, wortmannin decreased p110 activity, whereas ropivacaine had no significant effect on the activity of the four PI3K isoforms tested (fig. 3C).

To determine whether ropivacaine blocks Akt-dependent eNOS activation (and thereby prevents Src activation) specifically, Ca²⁺-dependent eNOS activation in the presence or absence of ropivacaine was assessed. HLMVECs were stimulated with the Ca²⁺ ionophore A23187 (5 μ M) for 10 min to activate eNOS *via* Ca²⁺/calmodulin¹⁰ and subsequently Src *via* nitric oxide signaling rather than Akt signaling¹⁰ (fig. 4). A23187 treatment induced a $45 \pm 34.8\%$ increase in Src activity compared with control ($P = 0.079$; fig. 4, A and B); however, this effect was not blocked by 1 μ M of ropivacaine ($P = 1$; fig. 4, A and B).

Ropivacaine Blocks Recruitment of p85 Regulatory Subunit of PI3K to TNF-R1

The recruitment of p85 regulatory subunit of PI3K to TNF-R1 is a key step leading to PI3K activation on binding of TNF α to TNF-R1.¹³ To detect the recruitment of p85, TNF-R1 was immunoprecipitated from HLMVEC lysates which were briefly (10 min) stimulated with TNF α in the absence or presence of ropivacaine at 1 nM. Western blots of the immunoprecipitated protein (fig. 5A) showed a statistically significant 4.46 ± 1.92 -fold increase ($P = 0.002$) in co-immunoprecipitation of p85 and TNF-R1 compared with untreated cells (fig. 5B) which was attenuated by $64 \pm 14.6\%$ in presence of 1 nM ropivacaine ($P = 0.04$; fig. 5B).

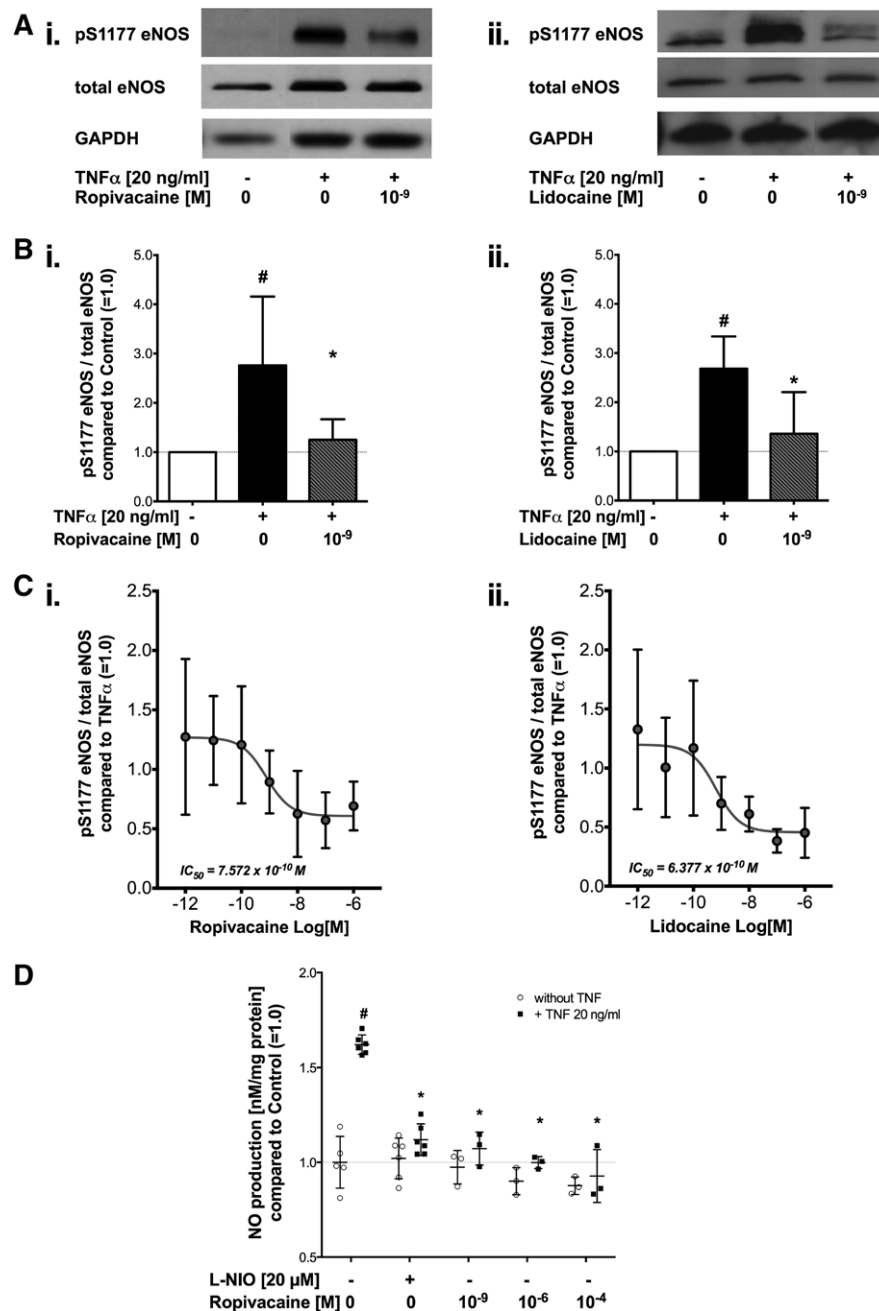


Fig. 2. Ropivacaine and lidocaine inhibit endothelial nitric oxide synthase (eNOS) phosphorylation and ropivacaine blocks tumor necrosis factor- α (TNF α)-induced nitric oxide (NO) production in human lung microvascular endothelial cells (HLMVEC). (A) Representative Western blots of eNOS phosphorylated at serine 1177 (pS1177 eNOS, row 1), total eNOS (row 2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, row 3) after treatment with TNF α (20 ng/ml) in the absence or presence of (i) ropivacaine (1 nM) or (ii) lidocaine (1 nM) for 20 min. (B) Quantitative analysis of densitometry of Western blots showing the ratio of pS1177 eNOS over total eNOS in HLMVEC treated with TNF α (20 ng/ml) in the absence or presence of (i) ropivacaine (1 nM) or (ii) lidocaine (1 nM) compared with untreated cells (set as 1.0). Data shown are mean \pm SD ($n = 6$); # $P < 0.05$ versus untreated cells, * $P < 0.05$ compared with TNF α alone. (C) Dose-response curve of Western blot densitometry data showing the ratio of pS1177 eNOS over total eNOS in HLMVEC treated with TNF α (20 ng/ml) in the absence or presence of log concentrations of (i) ropivacaine (1 pM to 1 μ M) or (ii) lidocaine (1 pM to 1 μ M) compared with TNF α alone (set as 1.0). Data shown are mean \pm SD ($n = 6$ –8 for ropivacaine and $n = 5$ –6 for lidocaine). (D) Assessment of cumulative NO production via chemiluminescent detection of nitrite ion (NO $_2^-$) directly proportional to NO in HLMVEC culture supernatants after treatment for 1 h with ropivacaine (1 nM, 1 μ M, 100 μ M) or the nonselective NO-synthase inhibitor N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) (20 μ M) in the absence (empty circles) or presence (black squares) of TNF α (20 ng/ml). NO production from untreated cells was set as 1.0. Data shown are mean \pm SD; $n = 3$ per group, except for TNF α alone and data with L-NIO, where $n = 6$. # $P < 0.05$ versus untreated cells, * $P < 0.05$ compared with TNF α alone.

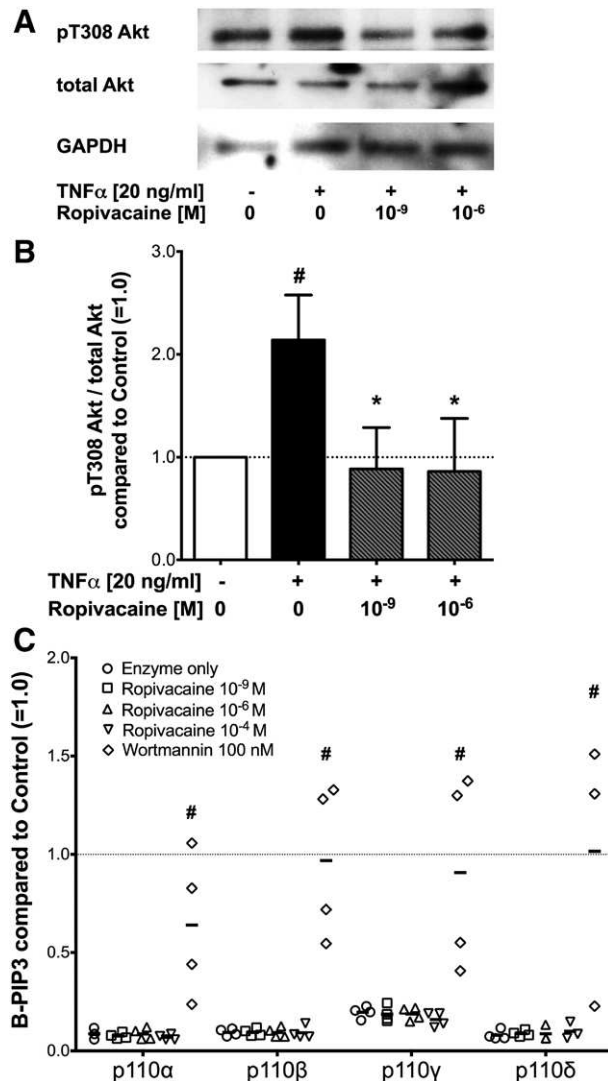


Fig. 3. Ropivacaine blocks activation of Akt kinase (Akt) upstream of endothelial nitric oxide synthase (eNOS) but does not directly inhibit phosphatidylinositol 3-kinase (PI3K) upstream of Akt. (A) Representative Western blot of Akt phosphorylated at threonine 308 (pT308 Akt, row 1), total Akt (row 2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, row 3) after treatment with tumor necrosis factor α (TNF α , 20 ng/ml) in the absence or presence of ropivacaine (1 nM, 1 μ M) for 20 min. (B) Quantitative analysis of densitometry of Western blots showing the ratio of pT308 Akt over total Akt in human lung microvascular endothelial cells treated with TNF α (20 ng/ml) in the absence (black bar) or presence (striped gray bars) of ropivacaine (1 nM, 1 μ M) compared with untreated cells (set as 1.0). Data shown are mean \pm SD (n = 7 per group); #P < 0.05 versus untreated cells, *P < 0.05 compared with TNF- α alone. (C) PI3K activity measured by enzyme-linked immunosorbent assay of biotinylated phosphatidylinositol (3,4,5)-triphosphate (B-PIP3) after incubation of different isoforms of p110 catalytic subunit of PI3K (α , β , γ , δ) with phosphatidylinositol (4,5)-bisphosphate (PIP2) substrate and ropivacaine (1 nM, 1 μ M, 100 μ M) or wortmannin (100 nM). Generated PIP3 competes with added B-PIP3 for binding, thus a high signal indicates low enzyme activity. Data shown are mean \pm SD (n = 4 per group); #P < 0.05 versus all other groups.

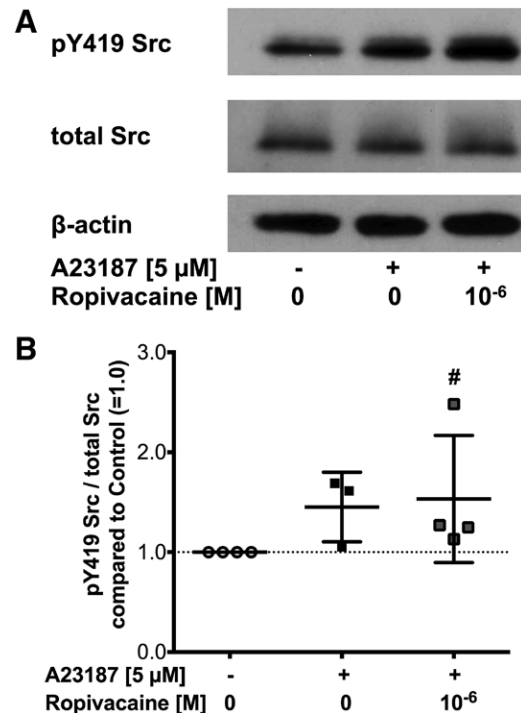


Fig. 4. Ropivacaine does not block Src activation induced by the non-Akt-dependent endothelial nitric oxide synthase activator calcium ionophore (A23187). (A) Representative Western blot of pY419 Src (row 1), total Src (row 2), and β -actin (row 3) after treatment with the non-Akt-dependent endothelial nitric oxide synthase activator calcium ionophore (A23187, 5 μ M) in the absence or presence of ropivacaine (1 nM, 1 μ M) for 10 min. (B) Quantitative analysis of densitometry of Western blots showing the ratio of pY419 Src over total Src after stimulation with A23187 alone (black squares), A23187 with ropivacaine (1 nM, 1 μ M, gray squares), or vehicle-treated cells (empty circles, set as 1.0) for 10 min. Data shown are mean \pm SD (n = 4); #P < 0.05 versus vehicle.

Ropivacaine Inhibits Src-dependent Phosphorylation of ICAM-1 and Neutrophil Adhesion to Endothelial Cells

Src-dependent phosphorylation of ICAM-1 is known to increase leukocyte adhesion in endothelial cells.¹⁶ We therefore first assessed whether ropivacaine inhibits TNF α -induced ICAM-1 phosphorylation in our cell culture model. HLMVEC lysates, incubated with different concentrations of ropivacaine (1 nM, 1 μ M) in the absence or presence of TNF α , were analyzed *via* Western blot, probing for phosphorylated ICAM-1 (at tyrosine 512, pY512 ICAM-1), and total ICAM-1 (fig. 6A). Densitometry analysis revealed that there was no alteration in basal pY512 ICAM-1 level in presence of ropivacaine alone ($P = 0.537$, data not shown). However, when applied in combination with TNF α , ICAM-1 phosphorylation observed in response to TNF α alone ($175 \pm 94.2\%$ compared with untreated cells; $P = 0.007$) was reduced by $45 \pm 23\%$ in presence of 1 nM ropivacaine ($P = 0.107$) and was completely blocked by 1 μ M ($P = 0.006$; fig. 6B). To assess the functional significance of the observed inhibition of ICAM-1 phosphorylation,

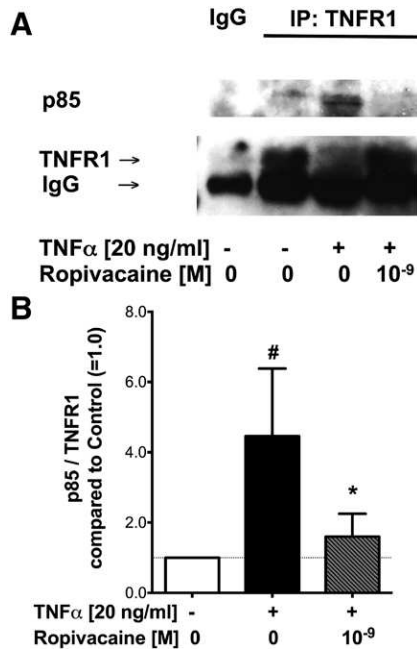


Fig. 5. Ropivacaine blocks recruitment of p85 regulatory subunit of phosphatidylinositol-3 kinase to tumor necrosis factor-receptor-1 (TNF-R1). (A) Representative Western blots of p85 regulatory subunit of phosphatidylinositol-3 kinase (row 1) and TNF-R1 (row 2) after immunoprecipitation of TNF-R1 from human lung microvascular endothelial cells treated with TNF α (20 ng/ml) in the absence or presence of ropivacaine (1 nM) for 10 min. Normal mouse immunoglobulin G (IgG) served as negative control for immunoprecipitation (lane 1). (B) Quantitative analysis of densitometry of Western blots from coimmunoprecipitation showing the ratio of p85 over TNF-R1 in human lung microvascular endothelial cells treated with TNF α (20 ng/ml) for 10 min in the absence (black bar) or presence (striped gray bar) of ropivacaine (1 nM) compared with untreated cells (white bar, set as 1.0). Data shown are mean \pm SD (n = 6 per group). #P < 0.05 versus untreated cells, *P < 0.05 compared with TNF α alone.

we quantified neutrophil adhesion to HLMVEC monolayers. Before the addition of TNF α (20 ng/ml) \pm ropivacaine (1 nM, 1 μ M, 100 μ M) for 20 min, HLMVECs were stimulated with lipopolysaccharide (4 μ g/ml) for 4 h to increase ICAM-1 expression as shown in the Western blot in figure 6Ci. Quantification of adherent, fluorescently labeled human neutrophils revealed a $63 \pm 27.6\%$ increase in adhesion in cells treated with TNF α for 20 min. Analysis with two-way ANOVA revealed a significant effect of both TNF α ($F(1, 56) = 14.14$; $P < 0.001$) and ropivacaine ($F(3, 56) = 6.765$; $P < 0.001$) and a significant interaction term between these two factors ($F(3, 56) = 5.177$; $P < 0.001$). *Post hoc* comparison between the values obtained from incubation with TNF α alone and ropivacaine at different concentrations incubated together with TNF α revealed a dose-dependent inhibition of neutrophil adhesion by increasing ropivacaine concentrations (statistically significant decrease at a concentration of 1 nM ropivacaine [$P = 0.01$] and higher; fig. 6Cii).

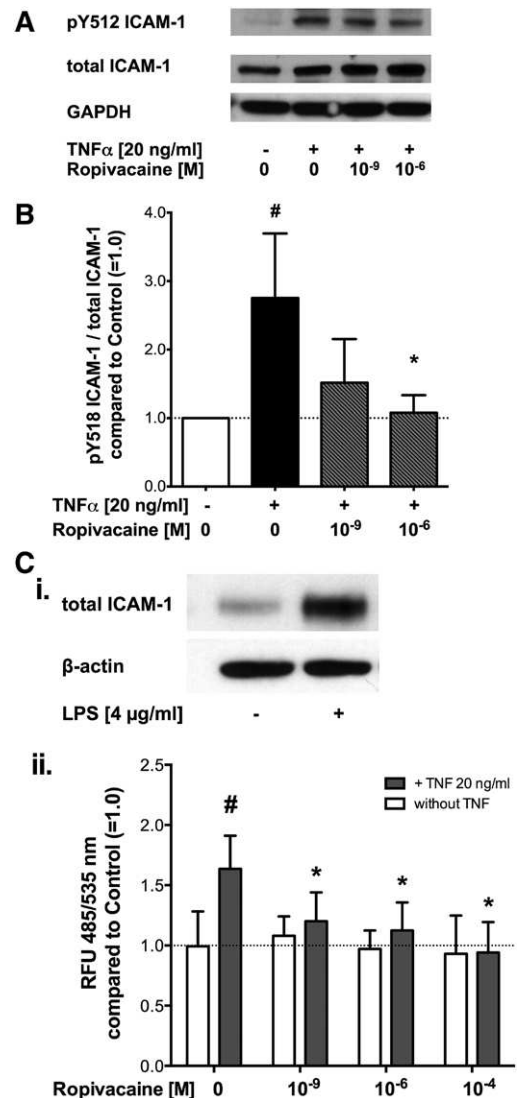


Fig. 6. Ropivacaine inhibits Src-dependent phosphorylation of intercellular adhesion molecule-1 (ICAM-1) and attenuates neutrophil adhesion to endothelial cells. (A) Representative Western blot of ICAM-1 phosphorylated at tyrosine 512 (pY512 ICAM-1, row 1), total ICAM-1 (row 2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin (row 3) after treatment with tumor necrosis factor α (TNF α , 20 ng/ml) in the absence or presence of ropivacaine (1 nM, 1 μ M) for 20 min. (B) Quantitative analysis of densitometry from Western blots showing the ratio of pY512 ICAM-1 over total ICAM-1 in human lung microvascular endothelial cells treated with TNF α (20 ng/ml) in the absence (black bar) or presence (striped gray bars) compared with untreated cells (white bar, set as 1.0). Data shown are mean \pm SD (n = 6 per group); #P < 0.05 versus untreated cells, *P < 0.05 compared with TNF α alone. (C) (i) Representative Western blot of ICAM-1 (row 1) and β -actin (row 2) after treatment with bacterial lipopolysaccharide (LPS, 4 μ g/ml) for 4 h. (ii) Quantification of adhesion of fluorescently labeled human neutrophils to human lung microvascular endothelial cells (pretreated with LPS [4 μ g/ml] for 4 h) followed by stimulation with TNF α (20 ng/ml) in the absence or presence of ropivacaine (1 nM, 1 μ M, 100 μ M). Data shown (relative fluorescence units [RFU]) are mean \pm SD (n = 8 per group); #P < 0.05 versus untreated cells, *P < 0.05 compared with TNF α alone.

Ropivacaine Inhibits Src-dependent Phosphorylation of Caveolin-1 and Disruption of Endothelial Monolayer Integrity

Src-dependent phosphorylation of caveolin-1 tyrosine 14 (pY14 Cav-1) leads to endothelial barrier dysfunction.⁸ Incubation of HLMVEC with ropivacaine at different concentrations for 20 min did not alter basal pY14 Cav-1 ($P = 0.311$, data not shown). As shown in figure 7, A and B, TNF α induced a $36 \pm 46\%$ increase in caveolin-1 phosphorylation, which was not considered to be statistically significant ($P = 1$). However, in the presence of 1 nM ropivacaine, we did not observe a significant increase in TNF α -induced caveolin-1 phosphorylation compared with untreated cells ($P = 0.4522$) but saw a reduction of $35 \pm 18\%$ compared with TNF α alone ($P = 0.012$; fig. 7, A and B).

Endothelial barrier disruption induced by TNF α (*i.e.*, decrease in monolayer integrity which is thought to represent an increase in paracellular permeability)²⁵ was evaluated by measuring TER of HLMVEC monolayers grown on gold-film electrodes. HLMVECs were treated with TNF α (20 ng/ml), ropivacaine (1 μ M), TNF α + ropivacaine, TNF α + NOS-inhibitor L-NIO (20 μ M, pretreatment for 1 h before addition of TNF α), TNF α + Src-inhibitor PP2 (10 μ M, pretreatment for 30 min before addition of TNF α), or were left untreated (control). TER was measured over time and normalized to baseline values as shown in figure 7C. TNF α decreased monolayer resistance at 6 h (fig. 7D) by $57 \pm 20\%$ ($P < 0.001$) compared with untreated monolayers, respectively. Coincubation of cells with TNF α and 1 μ M of ropivacaine significantly blocked the decrease in TER induced by TNF α alone by $42 \pm 19.3\%$ at 6 h ($P = 0.006$). As positive controls, pretreatment of monolayers with NOS and Src inhibitors L-NIO and PP2, respectively, was shown to also significantly attenuate the TNF α -induced decrease in TER at 6 h ($P < 0.001$ for L-NIO, $P = 0.049$ for PP2).

Discussion

Inflammatory vascular hyperpermeability is mediated in part by Src-dependent signaling,^{8,14–17,29,30} and thus Src may be a therapeutic target in the pathogenesis of inflammatory diseases such as acute lung injury, ARDS, cancer, and diabetes mellitus.^{1,3,4,7,18,22} In the current study, we characterized the molecular mechanism by which the amide-linked local anesthetics ropivacaine and lidocaine block inflammatory Src signaling induced by TNF α . In functional studies in which human endothelial cells were used, we observed that TNF α -induced neutrophil binding and loss of endothelial monolayer integrity could be abolished in cells simultaneously treated with ropivacaine. The concentrations of ropivacaine and lidocaine that were effective (1 nM to 1 μ M) are clinically relevant and consistent with a recent study which reported a well-tolerated, mean (total) maximal plasma concentration of 1.82 mg/l (5.5 μ M) during continuous epidural infusions of 0.2% ropivacaine at a rate of 10 mg/h for 48 h after major abdominal or urologic surgery.³¹

Previous studies have also shown a protective effect of local anesthetics on endothelial cell viability and function.^{32,33} However, these studies primarily focused on the inhibition of effects of prolonged (>24 h) cytokine exposure and used concentrations of local anesthetics up to 100,000 times higher than that used in the current study^{32,33} although still much lower than concentrations required to achieve effective blockade of voltage-gated sodium channels (IC₅₀ of 50 to 100 μ M).^{34,35} Interestingly, in an earlier study, we observed that inhibition of TNF α -induced Src activation by local anesthetics occurs independently of sodium channel blockade.²² Due to their lipophilic nature, local anesthetics distribute into cell membranes, affect their dynamics,³⁶ and interfere with the function of membrane-associated proteins like the G-protein-coupled m1 muscarinic acetylcholine receptor,³⁷ also at a much lower concentration (the IC₅₀ for lidocaine was 18 nM) than that which is necessary for sodium channel inhibition. Uniquely, the current report focused on the effect of amide-linked local anesthetics on rapidly appearing TNF α -induced signaling events known to be activated during the initial phase of vascular inflammation.¹⁶ We demonstrated specific inhibition of agonist (TNF α)-induced activation of signaling enzymes, but saw no effect on basal signaling, which might also be a possible explanation for the low IC₅₀ values observed.

The mechanism(s) that account for the now well-established anti-inflammatory as well as anti-metastatic actions of local anesthetics requires further investigation.^{18,22} There are structural similarities between ropivacaine (and lidocaine) and commercially available Src kinase inhibitors such as the combined Bcr-Abl/Src inhibitor Dasatinib[®] (Bristol-Myers Squibb Company, Princeton, NJ).^{38,39} Both types of drugs share an amide-linked phenyl group with substitution at positions 2 and 6, whereas the phenyl ring in local anesthetics shows two methyl groups and Dasatinib[®] one methyl and one chloride.^{39,40} Interestingly, this particular part of Dasatinib[®] is important in the mechanism of inhibition, as it binds (and therefore blocks) to the hydrophobic pouch of the adenosine triphosphate binding pocket within the kinase.³⁸ Our investigations, however, indicate that ropivacaine does not act as a competitive inhibitor of adenosine triphosphate binding in the Src kinase active site, that is, the common target of most kinase inhibitors.³⁸ To address other possible targets of local anesthetics such as ropivacaine and lidocaine, we interrogated potential signaling intermediates known to play a role in TNF α -induced Src activation.

Src activity in cultured cells and tissues is often assessed by measuring the phosphorylation status of active-site Src tyrosine 419 and inhibitory tyrosine 529 residues.⁸ eNOS activation and nitric oxide generation play an important role in Src activation¹⁰ and were significantly inhibited by ropivacaine and lidocaine in our experiments. A reduction of (lipopolysaccharide-induced) nitric oxide production by lidocaine in bovine aortic endothelial cells has previously

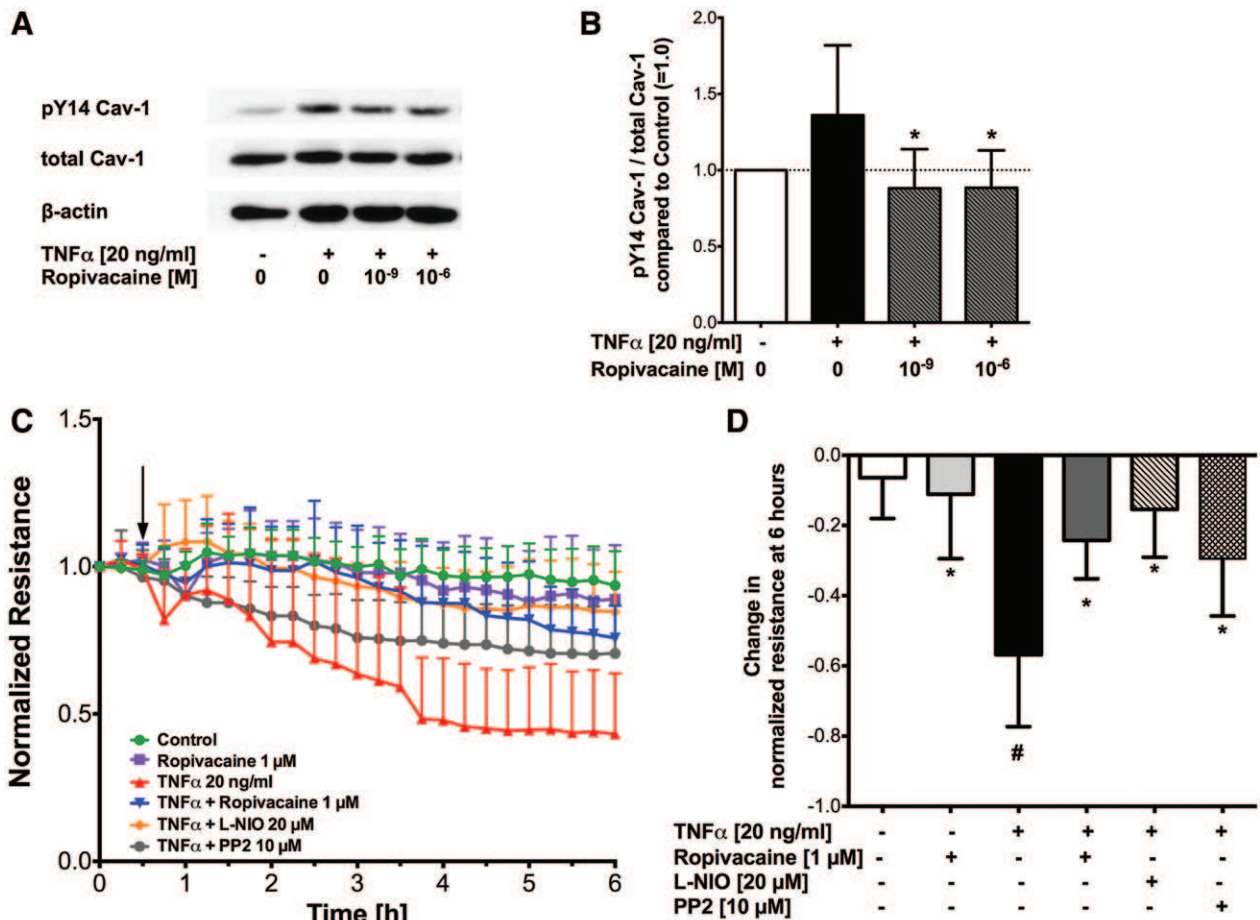


Fig. 7. Ropivacaine inhibits tumor necrosis factor- α (TNF α)-induced Src-dependent phosphorylation of caveolin-1 and attenuates endothelial barrier disruption. (A) Representative Western blots of caveolin-1 phosphorylated at tyrosine 14 (pY14 Cav-1, row 1), total Cav-1 (row 2), and β -actin (row 3) after treatment with TNF α (20 ng/ml) in the absence or presence of ropivacaine (1 nM, 1 μ M) for 20 min. (B) Quantitative analysis of densitometry of Western blots showing the ratio of pY14 Cav-1 over total Cav-1 in human lung microvascular endothelial cells treated with TNF α (20 ng/ml) in the absence (black bar) or presence (striped gray bars) of ropivacaine compared with untreated cells (white bar, set as 1.0). Data shown are mean \pm SD ($n = 8$); $*P < 0.05$ compared with TNF α alone. (C) Transendothelial electrical resistance of endothelial monolayers over time (normalized to baseline values). After 30-min equilibration, cells were incubated (arrow indicates beginning of treatment) with either TNF α (20 ng/ml, red), ropivacaine (1 μ M, purple), TNF α + ropivacaine (blue), TNF α + nitric oxide synthase inhibitor N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) (yellow, pretreatment for 1 h before addition of TNF α), TNF α + Src-inhibitor PP2 (gray, pretreatment for 30 min before addition of TNF α), or vehicle (control, green). Data shown are mean \pm SD of normalized resistance ($n = 7$ per group). (D) Quantification of decrease in normalized resistance at 6 h. Data shown are mean \pm SD; untreated cells (white bars), ropivacaine at 1 μ M (light gray bars), TNF α alone (black bar), TNF α + ropivacaine 1 μ M (dark gray bars), and TNF α + L-NIO (striped bars), TNF α + PP2 (checkered bars); $n = 7$ per group; # $P < 0.05$ versus untreated cells, $*P < 0.05$ compared with TNF α alone.

been reported, and the authors provided some evidence that the observed inhibition might (in part) have been due to a suppression of L-arginine uptake by the cells.⁴¹ Nitric oxide-dependent S-nitrosation of Src at cysteine 498 has also been reported not only to increase Src activity but also to lead to the disruption of cell-cell junctions.⁹

Akt protein kinase is known to activate eNOS *via* direct phosphorylation of serine 1177.^{42,43} Our studies demonstrate no effect of ropivacaine on Src activation induced by Ca²⁺ ionophore, a non-Akt-dependent activator of eNOS, but inhibited TNF α -induced Akt activation suggesting the site of ropivacaine action may be upstream of

Akt, for example *via* inhibition of PI3K. PI3Ks are divided into three different classes, class I, II, and III, and class I PI3K consists of two subgroups, IA and IB.⁴⁴ The class I PI3K isoforms function as heterodimers of the regulatory p85 subunit (of which there are two isoforms, p85 α and p85 β) and the catalytic p110 subunit (α , β , δ isoforms in class IA; γ isoform in class IB).⁴⁵ PI3K phosphorylates the 3'-OH residue of the inositol ring of phosphatidylinositol-(4,5) phosphate (PIP2) to generate PIP3, a lipid second messenger that has been shown to activate Akt and phosphoinositide-dependent kinase-1.^{44,46} By using the principle of PIP3 generation by PI3K in an *in vitro* kinase

assay, we showed that ropivacaine does not directly inhibit PI3K activity.

An alternative pathway leading to Src activation on stimulation with TNF α might be the generation of superoxide anions (O $_2^-$), for example by nicotinamide adenine dinucleotide phosphate-oxidase (NOX) *via* a pathway involving the activation of protein kinase C- ζ downstream of PI3K (fig. 8, gray arrows) as the stimulation of endothelial cells with TNF α was shown to induce a PI3K-dependent rapid increase in protein kinase C- ζ activation.^{16,47} Protein kinase C- ζ -mediated phosphorylation of p47^{phox} is thought to initiate the assembly and activation of the NOX complex.⁴⁸ The oxidants produced have been shown to activate Src as well.^{49,50}

The results of the current study, however, indicate that ropivacaine blocks TNF α -induced Src activation upstream of PI3K. On the binding of TNF α to its primary receptor in endothelial cells, TNF-R1, p85 is recruited to the receptor which leads to the assembly of the p85 and p110 heterodimer and thereby to activation of PI3K¹³ (fig. 8). Interestingly, we observed a diminished recruitment of p85 to TNF-R1 by ropivacaine in co-immunoprecipitation experiments of TNF-R1 and p85 in HLMVEC suggesting that ropivacaine may block TNF α signaling by disrupting TNF-R1-mediated PI3K activation, Akt phosphorylation, and eNOS-dependent nitric oxide production.

The initiation of transmembrane signaling *via* TNF-R1 includes oligomerization of ligand-receptor complexes,⁵¹ and there is some evidence that certain anesthetics (as shown for the less common local anesthetic phenylethanol) are able to inhibit oligomerization of transmembrane proteins.⁵² In addition, it has been shown that amide-linked local anesthetics alter membrane fluidity as well as conformation of membrane proteins.^{53,54} Thus, on the basis of the results of the current study, we speculate that inhibition of p85 recruitment might be due to alteration in the conformation of TNF-R1 or attenuation of its ability to oligomerize to initiate signaling across the membrane.⁵¹

In endothelial cells, we previously demonstrated that TNF α -induced Src activation leads to ICAM-1 tyrosine 512 phosphorylation which, by promoting actin-mediated ICAM-1 clustering, increases the avidity of neutrophil binding during the rapid, initial phase of inflammation.¹⁶ Subsequently, this rapid increase in endothelial cell adhesiveness leads to accelerated recruitment of circulating neutrophils and development of inflammation.¹⁶ Ropivacaine was shown to inhibit TNF α -induced Src-dependent ICAM-1 phosphorylation as well as neutrophil adhesion to monolayers of HLMVECs, thus supporting the hypothesis that ropivacaine has beneficial anti-inflammatory effects by blunting TNF α -mediated endothelial cell activation.

Src-dependent caveolin-1 tyrosine 14 phosphorylation also contributes to the development of inflammatory vascular hyperpermeability.^{8,29,30} In response to thrombin or reactive oxygen species (H $_2$ O $_2$), that is, agents that induce

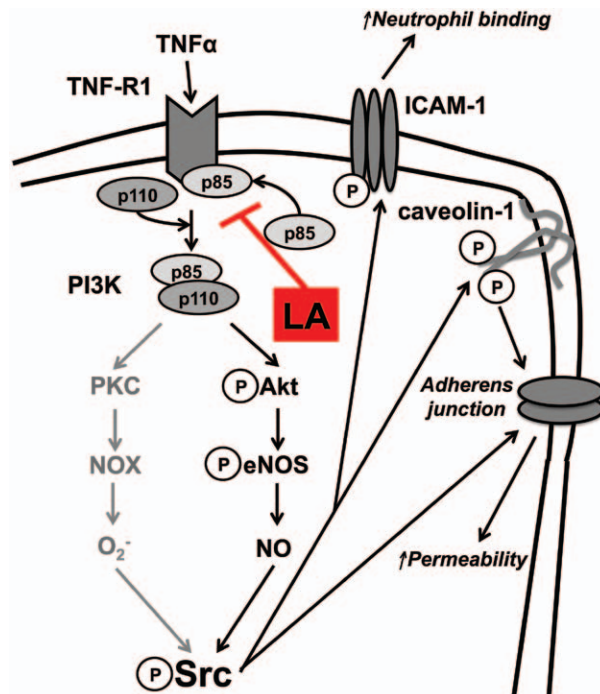


Fig. 8. Summary of proposed mechanism of inhibition of inflammatory Src signaling by local anesthetics. Local anesthetics (LA) inhibit recruitment of p85 regulatory subunit (p85) of phosphatidylinositol-3 kinase (PI3K) to tumor necrosis factor- α (TNF α) receptor 1 (TNF-R1), thus preventing PI3K activation and subsequent signaling involving phosphorylation/activation of Akt, endothelial nitric oxide synthase (eNOS), nitric oxide (NO) generation, and Src activation. Inhibition of Src-dependent intercellular adhesion molecule-1 (ICAM-1) phosphorylation blocks the increase in neutrophil binding, and the inhibition of direct and indirect (*via* the phosphorylation of caveolin-1) Src-dependent destabilization of adherens junctions attenuates endothelial barrier disruption and hyperpermeability. An alternative pathway (*gray arrows*) leading to Src activation might involve protein kinase C (PKC), nicotinamide adenine dinucleotide phosphate-oxidase (NOX), and the generation of superoxide anions (O $_2^-$).

the disruption of endothelial monolayer integrity,^{29,55} phosphorylated caveolin-1 promotes disruption of vascular endothelial-cadherin/catenin interactions,²⁹ perhaps by binding to catenins directly.⁵⁶ Disruption of cadherin/catenin interactions thought to be essential in the regulation of adherens junction stability,^{57,58} leads to disassembly of these cell-cell junctions and thereby an increase in transcellular endothelial permeability.⁵⁶ Alternatively, active Src may have directly modified vascular endothelial-cadherin in response to TNF α as it was shown that Src phosphorylation of vascular endothelial-cadherin tyrosine residues 658 and 685 in venous endothelial cells⁵⁹ promotes vascular endothelial-cadherin internalization, ubiquitination, and degradation, ultimately resulting in endothelial barrier disruption and increased vascular permeability.^{58,59}

Local anesthetic-mediated inhibition of Src-dependent ICAM-1 and caveolin-1 phosphorylation may be a potential mechanism for blocking endothelial barrier disruption

during acute inflammation. In addition, inhibition of TNF α -induced PI3K activation by ropivacaine upstream of Src might also be clinically relevant. In a recent study, it was shown that the phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 1 (P-Rex1) mediates TNF α -induced endothelial barrier disruption.²⁵ As ropivacaine inhibits TNF-R1-mediated PI3K activation, it should also decrease PIP3 generation and attenuate P-Rex1 activation⁶⁰ and signaling.²⁵

In conclusion, this study illustrates a novel anti-inflammatory mechanism of action of the local anesthetics ropivacaine and lidocaine. We demonstrate that in addition to the well-described action of Na⁺ channel blockade, local anesthetics also blocked TNF α -induced TNF-R1-dependent PI3K, Akt, and eNOS activation resulting in the inhibition of nitric oxide-driven Src signaling. We also showed that the inhibition of Src activation by the local anesthetics blocked TNF α -induced neutrophil adhesion and endothelial barrier disruption (inflammatory hyperpermeability). Thus, local anesthetics may have significant therapeutic benefit by attenuating vascular inflammation which we predict would minimize microvascular endothelial injury and inflammatory hyperpermeability associated with the pathogenesis of acute lung injury, ARDS, diabetes mellitus, and cancer.

Acknowledgments

This work was supported, in part, by the Society of Cardiovascular Anesthesiologists Foundation/International Anesthesia Research Society Starter Grant, Richmond, Virginia, (to Dr. Piegeler), U.S. Department of Defense research support W911NF-07-R-0003-04, Washington, D.C., and American Heart Association grant 09SDG2250933, Dallas, Texas (to Dr. Bonini), European Society of Anaesthesiology Research Grant, Brussels, Belgium (to Dr. Borgeat), and National Institutes of Health Heart Lung and Blood Institute grants R01 HL071626 and P01 HL060678, Bethesda, Maryland (to Dr. Minshall).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Minshall: University of Illinois at Chicago, 835 S. Wolcott Avenue (m/c 868), Room E417 MSB, Chicago, Illinois 60612. rminsh@uic.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

- Matthay MA, Ware LB, Zimmerman GA: The acute respiratory distress syndrome. *J Clin Invest* 2012; 122:2731–40
- Matthay MA, Zemans RL: The acute respiratory distress syndrome: Pathogenesis and treatment. *Annu Rev Pathol* 2011; 6:147–63
- Yang SJ, Chen CY, Chang GD, Wen HC, Chen CY, Chang SC, Liao JF, Chang CH: Activation of Akt by advanced glycation end products (AGEs): Involvement of IGF-1 receptor and caveolin-1. *PLoS One* 2013; 8:e58100
- Gillrie MR, Krishnegowda G, Lee K, Buret AG, Robbins SM, Looareesuwan S, Gowda DC, Ho M: Src-family kinase dependent disruption of endothelial barrier function by *Plasmodium falciparum* merozoite proteins. *Blood* 2007; 110:3426–35
- Guarino M: Src signaling in cancer invasion. *J Cell Physiol* 2010; 223:14–26
- Johnson ER, Matthay MA: Acute lung injury: Epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv* 2010; 23:243–52
- Bhattacharya J, Matthay MA: Regulation and repair of the alveolar-capillary barrier in acute lung injury. *Annu Rev Physiol* 2013; 75:593–615
- Hu G, Minshall RD: Regulation of transendothelial permeability by Src kinase. *Microvasc Res* 2009; 77:21–5
- Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M: S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. *J Biol Chem* 2010; 285:3806–14
- Chen Z, Bakhshi FR, Shajahan AN, Sharma T, Mao M, Trane A, Bernatchez P, van Nieuw Amerongen GP, Bonini MG, Skidgel RA, Malik AB, Minshall RD: Nitric oxide-dependent Src activation and resultant caveolin-1 phosphorylation promote eNOS/caveolin-1 binding and eNOS inhibition. *Mol Biol Cell* 2012; 23:1388–98
- Mao M, Sudhakar V, Ansenberger-Fricano K, Fernandes DC, Tanaka LY, Fukai T, Laurindo FR, Mason RP, Vasquez-Vivar J, Minshall RD, Stadler K, Bonini MG: Nitroglycerin drives endothelial nitric oxide synthase activation *via* the phosphatidylinositol 3-kinase/protein kinase B pathway. *Free Radic Biol Med* 2012; 52:427–35
- Kobayashi T, Nemoto S, Ishida K, Taguchi K, Matsumoto T, Kamata K: Involvement of CaM kinase II in the impairment of endothelial function and eNOS activity in aortas of Type 2 diabetic rats. *Clin Sci (Lond)* 2012; 123:375–86
- Pincheira R, Castro AF, Ozes ON, Idumalla PS, Donner DB: Type 1 TNF receptor forms a complex with and uses Jak2 and c-Src to selectively engage signaling pathways that regulate transcription factor activity. *J Immunol* 2008; 181:1288–98
- Hu G, Vogel SM, Schwartz DE, Malik AB, Minshall RD: Intercellular adhesion molecule-1-dependent neutrophil adhesion to endothelial cells induces caveolae-mediated pulmonary vascular hyperpermeability. *Circ Res* 2008; 102:e120–31
- Hu G, Ye RD, Dinanuer MC, Malik AB, Minshall RD: Neutrophil caveolin-1 expression contributes to mechanism of lung inflammation and injury. *Am J Physiol Lung Cell Mol Physiol* 2008; 294:L178–86
- Liu G, Vogel SM, Gao X, Javaid K, Hu G, Danilov SM, Malik AB, Minshall RD: Src phosphorylation of endothelial cell surface intercellular adhesion molecule-1 mediates neutrophil adhesion and contributes to the mechanism of lung inflammation. *Arterioscler Thromb Vasc Biol* 2011; 31:1342–50
- Liu G, Place AT, Chen Z, Brovkovich VM, Vogel SM, Muller WA, Skidgel RA, Malik AB, Minshall RD: ICAM-1-activated Src and eNOS signaling increase endothelial cell surface PECAM-1 adhesivity and neutrophil transmigration. *Blood* 2012; 120:1942–52
- Blumenthal S, Borgeat A, Pasch T, Reyes L, Booy C, Lambert M, Schimmer RC, Beck-Schimmer B: Ropivacaine decreases inflammation in experimental endotoxin-induced lung injury. *ANESTHESIOLOGY* 2006; 104:961–9
- Feng G, Liu S, Wang GL, Liu GJ: Lidocaine attenuates lipopolysaccharide-induced acute lung injury through

- inhibiting NF-kappaB activation. *Pharmacology* 2008; 81:32–40
20. Konrad CJ, Schuepfer GK, Neuburger M, Schley M, Schmelz M, Schmeck J: Reduction of pulmonary edema by short-acting local anesthetics. *Reg Anesth Pain Med* 2006; 31:254–9
 21. Schley MT, Casutt M, Haberthür C, Dusch M, Rukwied R, Schmelz M, Schmeck J, Schüpfer GK, Konrad CJ: Long-acting local anesthetics attenuate FMLP-induced acute lung injury in rats. *Anesth Analg* 2009; 109:880–5
 22. Piegeler T, Votta-Velis EG, Liu G, Place AT, Schwartz DE, Beck-Schimmer B, Minshall RD, Borgeat A: Antimetastatic potential of amide-linked local anesthetics: Inhibition of lung adenocarcinoma cell migration and inflammatory Src signaling independent of sodium channel blockade. *ANESTHESIOLOGY* 2012; 117:548–59
 23. Place AT, Chen Z, Bakhshi FR, Liu G, O'Bryan JP, Minshall RD: Cooperative role of caveolin-1 and C-terminal Src kinase binding protein in C-terminal Src kinase-mediated negative regulation of c-Src. *Mol Pharmacol* 2011; 80:665–72
 24. Hastie CJ, McLauchlan HJ, Cohen P: Assay of protein kinases using radiolabeled ATP: A protocol. *Nat Protoc* 2006; 1:968–71
 25. Naikawadi RP, Cheng N, Vogel SM, Qian F, Wu D, Malik AB, Ye RD: A critical role for phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 1 in endothelial junction disruption and vascular hyperpermeability. *Circ Res* 2012; 111:1517–27
 26. Sebaugh JL: Guidelines for accurate EC50/IC50 estimation. *Pharm Stat* 2011; 10:128–34
 27. Osaki M, Oshimura M, Ito H: PI3K-Akt pathway: Its functions and alterations in human cancer. *Apoptosis* 2004; 9: 667–76
 28. Schultze SM, Hemmings BA, Niessen M, Tschopp O: PI3K/AKT, MAPK and AMPK signalling: Protein kinases in glucose homeostasis. *Expert Rev Mol Med* 2012; 14:e1
 29. Sun Y, Hu G, Zhang X, Minshall RD: Phosphorylation of caveolin-1 regulates oxidant-induced pulmonary vascular permeability *via* paracellular and transcellular pathways. *Circ Res* 2009; 105:676–85
 30. Sun Y, Minshall RD, Hu G: Role of caveolin-1 in the regulation of pulmonary endothelial permeability. *Methods Mol Biol* 2011; 763:303–17
 31. Cusato M, Allegri M, Niebel T, Ingelmo P, Broglia M, Braschi A, Regazzi M: Flip-flop kinetics of ropivacaine during continuous epidural infusion influences its accumulation rate. *Eur J Clin Pharmacol* 2011; 67:399–406
 32. de Klaver MJ, Buckingham MG, Rich GF: Lidocaine attenuates cytokine-induced cell injury in endothelial and vascular smooth muscle cells. *Anesth Analg* 2003; 97:465–70
 33. de Klaver MJ, Weingart GS, Obrig TG, Rich GF: Local anesthetic-induced protection against lipopolysaccharide-induced injury in endothelial cells: The role of mitochondrial adenosine triphosphate-sensitive potassium channels. *Anesth Analg* 2006; 102:1108–13
 34. Scholz A, Kuboyama N, Hempelmann G, Vogel W: Complex blockade of TTX-resistant Na⁺ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. *J Neurophysiol* 1998; 79:1746–54
 35. Hollmann MW, Durieux ME: Local anesthetics and the inflammatory response: A new therapeutic indication? *ANESTHESIOLOGY* 2000; 93:858–75
 36. de Paula E, Schreiber S: Use of a novel method for determination of partition coefficients to compare the effect of local anesthetics on membrane structure. *Biochim Biophys Acta* 1995; 1240:25–33
 37. Hollmann MW, Fischer LG, Byford AM, Durieux ME: Local anesthetic inhibition of m1 muscarinic acetylcholine signaling. *ANESTHESIOLOGY* 2000; 93:497–509
 38. Schenone S, Bruno O, Radi M, Botta M: New insights into small-molecule inhibitors of Bcr-Abl. *Med Res Rev* 2011; 31:1–41
 39. Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K, Castaneda S, Cornelius LA, Das J, Doweyko AM, Fairchild C, Hunt JT, Inigo I, Johnston K, Kamath A, Kan D, Klei H, Marathe P, Pang S, Peterson R, Pitt S, Schieven GL, Schmidt RJ, Tokarski J, Wen ML, Wityak J, Borzilleri RM: Discovery of N-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* 2004; 47:6658–61
 40. Owen MD, Dean LS: Ropivacaine. *Expert Opin Pharmacother* 2000; 1:325–36
 41. Takaishi K, Kitahata H, Kawahito S: Local anesthetics inhibit nitric oxide production and L-arginine uptake in cultured bovine aortic endothelial cells. *Eur J Pharmacol* 2013; 704:58–63
 42. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999; 399:597–601
 43. Mineo C, Shaul PW: Regulation of eNOS in caveolae. *Adv Exp Med Biol* 2012; 729:51–62
 44. Karar J, Maity A: PI3K/AKT/mTOR pathway in angiogenesis. *Front Mol Neurosci* 2011; 4:51
 45. Fruman DA, Meyers RE, Cantley LC: Phosphoinositide kinases. *Annu Rev Biochem* 1998; 67:481–507
 46. Toker A, Cantley LC: Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* 1997; 387:673–6
 47. Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, Malik AB: Phosphatidylinositol 3-kinase gamma signaling through protein kinase C ζ induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. *J Biol Chem* 2006; 281:16128–38
 48. Dang PM, Fontayne A, Hakim J, El Benna J, Périain A: Protein kinase C zeta phosphorylates a subset of selective sites of the NADPH oxidase component p47phox and participates in formyl peptide-mediated neutrophil respiratory burst. *J Immunol* 2001; 166:1206–13
 49. Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y, Yazaki Y: Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 1997; 100:1813–21
 50. Meng TC, Fukada T, Tonks NK: Reversible oxidation and inactivation of protein tyrosine phosphatases *in vivo*. *Mol Cell* 2002; 9:387–99
 51. Lewis AK, Valley CC, Sachs JN: TNFR1 signaling is associated with backbone conformational changes of receptor dimers consistent with overactivation in the R92Q TRAPS mutant. *Biochemistry* 2012; 51:6545–55
 52. Anbazhagan V, Munz C, Tome L, Schneider D: Fluidizing the membrane by a local anesthetic: Phenylethanol affects membrane protein oligomerization. *J Mol Biol* 2010; 404:773–7
 53. Park JS, Jung TS, Noh YH, Kim WS, Park WI, Kim YS, Chung IK, Sohn UD, Bae SK, Bae MK, Jang HO, Yun I: The effect of lidocaine-HCl on the fluidity of native and model membrane lipid bilayers. *Korean J Physiol Pharmacol* 2012; 16:413–22
 54. Mojmudar EH, Lyubartsev AP: Molecular dynamics simulations of local anesthetic articaine in a lipid bilayer. *Biophys Chem* 2010; 153:27–35
 55. Siller-Matula JM, Schwameis M, Blann A, Mannhalter C, Jilka B: Thrombin as a multi-functional enzyme. Focus on *in vitro* and *in vivo* effects. *Thromb Haemost* 2011; 106:1020–33
 56. Kronstein R, Seebach J, Grossklaus S, Minten C, Engelhardt B, Drab M, Liebner S, Arsenijevic Y, Taha AA, Afanasieva

- T, Schnittler HJ: Caveolin-1 opens endothelial cell junctions by targeting catenins. *Cardiovasc Res* 2012; 93: 130–40
57. Dejana E, Giampietro C: Vascular endothelial-cadherin and vascular stability. *Curr Opin Hematol* 2012; 19:218–23
 58. Dejana E, Orsenigo F, Lampugnani MG: The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci* 2008; 121(Pt 13):2115–22
 59. Orsenigo F, Giampietro C, Ferrari A, Corada M, Galaup A, Sigismund S, Ristagno G, Maddaluno L, Koh GY, Franco D, Kurtcuoglu V, Poulidakos D, Baluk P, McDonald D, Grazia Lampugnani M, Dejana E: Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability *in vivo*. *Nat Commun* 2012; 3:1208
 60. Hill K, Krugmann S, Andrews SR, Coadwell WJ, Finan P, Welch HC, Hawkins PT, Stephens LR: Regulation of P-Rex1 by phosphatidylinositol (3,4,5)-trisphosphate and Gbetagamma subunits. *J Biol Chem* 2005; 280:4166–73